Remarks

The claims have been rewritten to set forth the critical features of the invention in producing glycosylated alpha 1-antitrypsin. It has been found that not only is the selectionof the strain of the yeast important but also the selection of a vector comprised of a particular plasmid. In the present case, the *P. pastoris* strains of KM71 and SMD1168H and plasmids $pGAP_z$ and $pPIC_z$ combination are selected.

The successful expression of recombinant genes in foreign organisms is not a predictable event. Many factors are involved such as the organism, the particular host within that organism, the promoter, additional upstream regulatory sequences, the codon usage in the gene for that organism, the length of the gene, the sequence in the downstream region including the termination area, the protease constituents of the host both within the cell and outside of the cell, the number of gene copies per cell and many other factors. In addition to these factors and their effects on their own, many of them interact and the combined effect of these many factors AND their interactions cannot be predicted with any degree of certainty. For example, one cannot predict with certainty that a given gene will express at a certain level when inserted into a given host with a given promoter, set of codons, terminator and at so many copies per cell. Essentially, one has to perform the experiment to determine whether any expression will occur under a given set of conditions (or a given combination of elements). If some level of expression is obtained, the level could be anywhere from very low to very high. In the former case, a given process could be commercially unacceptable whereas in the latter case, the

process might be viable. Once again, the mere fact that expression occurs with a given combination of elements may not guarantee expression at a commercially viable level.

The strain used by Kang et al is not proteinease deficient and the expression vector pHIL-D2AT is used.

There are a number of known factors involved in the replication of polypeptides in yeast. The most studied is the production of alpha 1-antitrypsin in *S. cerevisiae* and *S. pombe* employing different promoters. It was also found that the selection of a particular strain is necessary to achieve enhanced production (see U.S. Patent No. 4,752,576).

The production of polypeptides in *P. pastoris* is not well known. *P. pastoris* is capable of intra or extra-cellular production of polypeptides. The resulting product could be non-glycosylated or hyperglycosylated.

Previous attempts by applicants to produce alpha 1-antitrypsin and alpha 1-antichymotrypsin by transformation of pPIC9/x-antitrypsin and pPIC9/d-antichymotrypsin constructs into GS115/HIS4 defective strain failed to produce the desired products.

Duplication of the experiment described by Kang et al utilizing *P. pastoria* strain GS115 with a vector comprised of plasmid pGAPz and pPICz also failed. What was produced by extracellular secretion was a multiplicity of small molecular weight fragments.

The plasmid pH AT85 used with the strain of Kang et al and the presently claimed strains did not produce alpha 1-antitrypsin.

The fact that there are protease-deficient strains and non-protease deficient strains

of *P. pastoris* and that not all plasmids used are affected takes away from the presently claimed invention to be obvious.

Further, a critical feature of the invention resides in the pH of the fermentation process and the *P. pastoria* species.

There are many proteins produced utilizing *P. pastoris* which do not form a glycosylated product. Moreover, the processes involve shake-flask procedures. The accepted methods to produce glycosylated alpha-antitrypsin in shake-flask is according to Kang, et al and Zhang, et al. However, the processes cannot be carried over to large scale fermentation. These prior art studies report folded protein having no biological activity, non-glycosylated alpha 1-antitrypsin, hyperglycosylated alpha 1-antitrypsin and glycosylated alpha 1-antitrypsin.

In contrast, applicants found that alpha 1-antitrypsin production of pH 3.0, 4.0 and 5.0 cause degradation and that at pH 6.8 production is optimized.

Hyperglycosylation was also a problem in the prior art.

Initial attempts to obtain alpha 1-antitrypsin was with Invitrogen Corporation that has world-wide patents on the production of pharmaceuticals in *P. pastoris* yeast. The report discloses that there was difficulty following prior art processes even in a shake-flask method.

The University of Nebraska, which is considered to be the world experts in *P. pastoris* fermentation, had difficulties in obtaining alpha 1-antitrypsin using prior art methods in large fermentors. The problem faced was the degranulation of the product when performed at pH 3.0, 4.0 and 5.0 after 24 hours.

The problem was that the alpha 1-antitrypsin folded or degraded. Activity with elastase was detected but believed it was because of active segments of the enzyme. Moreover, over 21 strains of *P. pastoris* were tested but there was success in only four strains of the yeast. However, yield was small and according to the University of Nebraska, it was questionable whether or not the product was the glycoylated alpha 1-antitrypsin or an active segment of the protein when prepared according to Kang, et al and Zhang, et al.

It took over six months and $300,000 in costs to arrive at the species and pH for the process to work. The work was performed by experts on *P. pastoria* processes.

Consequently, the prior art references do not lead one in the art to a large scale production of biologically active glycosylated alpha 1-antitrypsin according to the invention.

Claims 13-17 have been allowed in the corresponding European Patent Application.

Reconsideration and favorable action are earnestly solicited.

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